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DECLARATION UNDER 37 C.F.R. §1.132

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Commissioner for Patents
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Sir:

We, Liang-Fong Wong and Nicholas Mazarakis, declare and state that:

1. A copy of our *curriculum vita* demonstrating our education, training and experience is appended hereto. We are employed by the Assignee of U.S. application Serial No. 09/701,014, and are familiar with the application and its prosecution history. We are considered by our peers to be experts in the field to which the application pertains, and are otherwise qualified to speak and render expert opinions as to the present application, invention, and issues of the Office Action dated March 20, 2003. Thus, this Declaration is in response to the Office Action.

2. The following experiments were performed by us or under our direction, supervision or control, and in the ordinary course of business.

Aim:

To test neuroprotective effects of EIAV.Bcl2 *in vivo* after injection of glutamate receptor agonists (Bk 1248, p147).

Introduction:

In order to test the effectiveness of EIAV vectors as gene therapy tools for cerebral ischemia, an excitotoxic model in the rat hippocampus was set up. During stroke, glutamate is released, inducing widespread cell death and neuronal degeneration in the brain. An *in vivo* model of this event was established by direct injection of glutamate receptor agonist into the hippocampus, one of the most vulnerable areas to ischemia. EIAV vectors expressing a control protein, β-galactosidase (pONY8.Z) and an anti-apoptotic molecule Bcl2 (pONY8.Bcl2Flag) were tested in this model.

Method:

Adult male Wistar rats were bilaterally transduced with either 3 μl pONY8Z/rabies or 3 μl pONY8.Bcl2/rabies into the CA1 region of the hippocampus. Approximately 1.5 x 10^6 transduction units of each vector were injected into the hippocampus. 2 weeks later, excitotoxic agents such as the glutamate receptor agonist N-methyl-D-aspartic acid (NMDA) was injected into the right hippocampus (0.5 μl of 5mM NMDA), while phosphate buffered saline (PBS) was injected into the contralateral hippocampus.

Experimental groups:

Group	Lentivector	Excitotoxic agent	
		Control (Left)	Insult (Right)
1 (n=4)	pONY8Z/rabies	PBS	5mM NMDA
2 (n=5)	pONY8.Bcl2Flag/rabies	PBS	5mM NMDA

72 hours after NMDA-induced excitotoxicity, animals were sacrificed by transcardial perfusion with 4% paraformaldehyde and the brains were extracted. Brains were post-fixed in 4% paraformaldehyde overnight at 4°C, after which they were cryoprotected in 30% sucrose for 3 days. 25 μm sections were obtained on the cryostat and histological analysis was performed. Immunohistochemistry was performed with NeuN antibody (Chemicon, UK) in a dilution of 1:300 in 10% normal goat serum (Vector Laboratories, UK). Following an overnight incubation in NeuN antibody, the sections were washed with PBS three times, 5 min each time, and secondary antibody detection using a biotinylated anti-mouse antibody

was performed according to manufacturer's instructions (Vector Laboratories, UK). VisualiZation of the immunostaining was enabled with DAB staining (Vector Laboratories, UK). Fluorojade-B staining was also performed according to manufacturer's instructions (Histo-chem Inc, USA).

Results:

Injection of 5mM NMDA (in a total volume of 0.5μl) into pONY8.Z transduced rat hippocampus induced a lesion in the CA1 layer, characterized by the loss of NeuN staining within CA1 neurons (Figure 1A). In addition, there was an increase in Fluorojade staining (Figure 1B) compared to the control PBS injection into the hippocampus (Figure 1C). In contrast, in pONY8.Bcl2Flag transduced animals, 5mM NMDA injection into the CA1 induced minimal damage, with many NeuN positive neurons surviving (Figure 1D). No appreciable increase in Fluorojade staining was observed after NMDA injection (Figure 1E) compared to PBS injection (Figure 1F).

The results were quantified by measuring the length of unlesioned and lesioned CA1 using the Axiovision software (Axiovision, UK). Multiple sections were analyzed for each sample (6-12) and the results were expressed as a percentage of CA1 lesion (over total CA1 length). Injection of PBS into the hippocampus did not cause any significant loss of CA1 neurons in either pONY8.Z transduced hippocampus or pONY8.Bcl2Flag transduced hippocampus (Figure 2). In contrast, only 24.0 ± 4.0% of CA1 survived after injection of 5mM NMDA in control pONY8.Z transduced animals, while 79.5 ± 9.3% of CA1 survived after 5mM NMDA injection in pONY8.Bcl2Flag transduced animals (Figure 2). This suggests that expression of Bcl2 in the hippocampus significantly protected these neurons from excitotoxic damage induced by 5mM NMDA ($P<0.001$, unpaired Students' t-test).

To confirm that the protection was mediated by lentiviral expression of Bcl2 in the hippocampus, immunohistochemistry with β-galactosidase and Bcl2 antibodies were performed in pONY8.Z- and pONY8.Bcl2Flag-transduced hippocampal sections respectively. β-galactosidase-positive neurons were detected in the CA1 layer of the hippocampus (Figure 3A); these neurons also expressed the NeuN marker. Approximately 47% of the CA1 layer was transduced. In the pONY8.Bcl2Flag-transduced hippocampus, Bcl2 expressing neurons could also be detected (Figure 3B).

Conclusion:

Injection of excitotoxic agents such as the glutamate receptor agonist NMDA into the CA1 layer of the hippocampus resulted in neuronal degeneration and cellular loss in control

animals (pONY8.Z transduced). In contrast, in pONY8.Bcl2Flag-transduced animals, lentiviral mediated expression of Bcl2, as detected by Bcl2 immunohistochemistry, protected these CA1 neurons from NMDA-induced excitotoxicity by approximately 55%. This suggests that the use of EIAV vectors encoding anti-apoptotic factors may be useful in gene therapy for stroke.

Figure Legends

Figure 1: NeuN immunohistochemistry and Fluorojade-B staining in hippocampal sections of pONY8.Z and pONY8.Bcl2Flag transduced animals. (A, D) NeuN staining of the hippocampus in pONY8.Z- and pONY8.Bcl2Flag transduced animals respectively. Injection of PBS into the hippocampus did not significantly affect the NeuN staining in the hippocampus. In pONY8.Z transduced hippocampus, injection of 5mM NMDA caused a significant decrease in NeuN staining in the CA1 layer (A). In contrast, 5mM NMDA did not reduce NeuN staining in pONY8.Bcl2Flag transduced hippocampus (D). (B, C) Fluorojade-B staining in the PBS- and the NMDA-injected hippocampus respectively in the pONY8.Z transduced animals. (E, F) Fluorojade-B staining in the PBS- and the NMDA-injected hippocampus respectively in the pONY8.Bcl2Flag transduced animals.

Figure 2: EIAV-mediated expression of Bcl2 protects the hippopcampus from NMDA-induced excitotoxicity. The CA1 lesions in pONY8.Z and pONY8.Bcl2Flag-transduced animals were measured and expressed as a percentage of the total CA1 length. Scale bar represents 200 μm.

Figure 3: Expression of β-galactosidase and Bcl2 in pONY8.Z and pONY8.Bcl2Flag transduced animals. (A) β-galactosidase immunohistochemistry was detected in hippocampal neurones located within the CA1 layer (green) while NeuN expressing cells are indicated in red. (B) In pONY8.Bcl2Flag transduced animals, Bcl2 immunohistochemistry was also detected in the hippocampal neurones. Scale bar represents 50 μm.

Figures

Figure 1

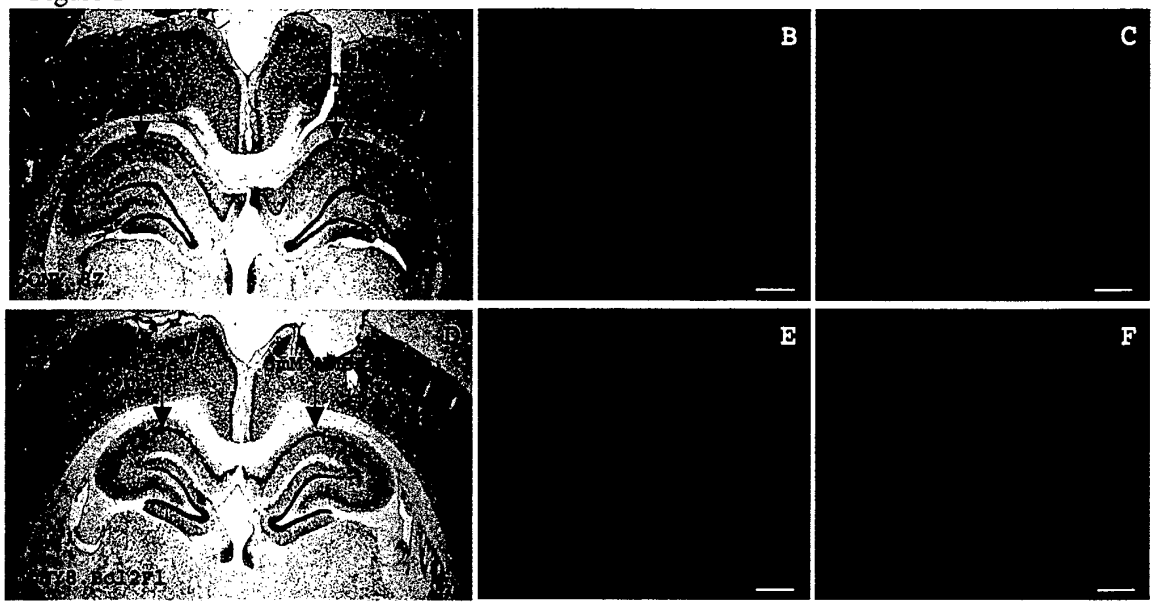


Figure 2

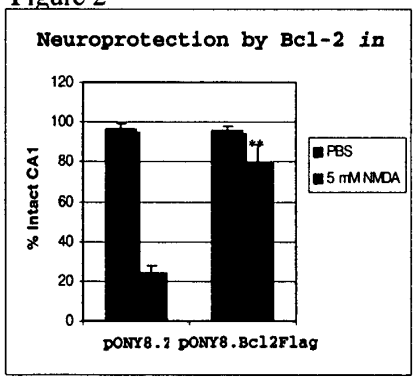
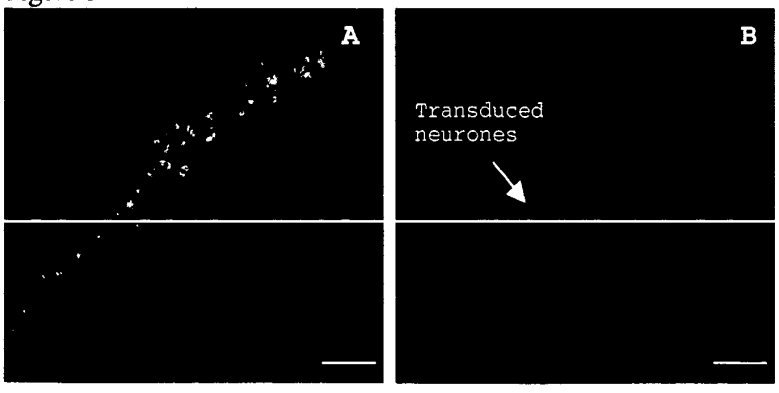


Figure 3



3. To summarize, taken together with the examples already presented in the instant application, these results demonstrate that expression of a neuroprotective gene in the hippocampus, via a rabies pseudotyped virus, protects hippocampal neurons from destruction in an *in vivo* stroke model. This use of the claimed composition is consistent with the teachings of the specification. Therefore, it is respectfully submitted that these data directly refute the enablement rejection of claim 31 under 35 U.S.C. §112, first paragraph, in the Office Action. Thus, reconsideration and withdrawal of the Section 112, first paragraph, rejection are respectfully solicited.

4. We further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true and further, that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 19.8.03

Nicholas Mazarakis

Date: 19.8.03

Liang-Fong Wong

Curriculum Vitae

Wong Liang-Fong

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Employment History

December 2002 – present
Oxford Biomedica (UK), Oxford
Scientist

May 2000 – September 2001
University Research Centre for Neuroendocrinology & Department of Physiology, University of Bristol
Postdoctoral research assistant (funded by Wellcome Trust project grant)

Educational History

September 1996 – April 2000
University Research Centre for Neuroendocrinology, University of Bristol
PhD degree; Thesis entitled "Gene transfer studies on central homeostatic mechanisms" (funded on Wellcome Prize Studentship)

July 1992 - July 1996
Department of Microbiology, National University of Singapore, 11 Kent Ridge Crescent Singapore 0511
Degree of Bachelor of Science (First Class Honours)

January 1990 - December 1991
Raffles Junior College, 53 Mount Sinai Road, Singapore 276880
GCE 'A' Levels (distinctions in Physics, Chemistry, Biology, Math) and GCE 'AS' Levels (distinction in General Paper, French and credit in Chinese)

January 1986 - December 1989
Raffles Girls' Secondary School, 20 Anderson Road, Singapore 259978
GCE '0' Levels (9 distinctions)

Academic Honours

1996-1999 • Overseas Research Student Award
1996 • Runme Shaw Gold Medal for best student in Microbiology in the B.Sc Examination (Honours)

1995 • Singapore National Academy of Science Award in Microbiology
• Runme Shaw Book Prize for best student in Microbiology in the Final B.Sc. Examination

1994 • Biochemistry Departmental Silver Medal for best student in Biochemistry

1993 • HongKong and Shanghai Banking Corporation Centenary Scholarship

Scientific Publications

***Wong L-F**, Polson J, Murphy D, Paton JFR and Kasparov S (2002)* Genetic and pharmacological dissection of pathways involved in the angiotensin II-mediated depression of baroreflex function. FASEB J 16:1595-601

***Wong L-F** and Murphy D (2002)* Adenoviral-mediated over-expression of Brn2 in the rat paraventricular nucleus: no effect on vasopressin or corticotrophin releasing factor RNA levels. Mol Cell Endocrinol (in press)

Hussain S, Assender JW, Bond M, ***Wong L-F**, Murphy D, Newby AC (2002)* Activation of protein kinase Cζ is essential for cytokine-induced metalloproteinase-1, -3, and -9 secretion from rabbit smooth muscle cells and inhibits proliferation. J Biol Chem 277(30):27345-52

Paton JFR, Deuchars J, Ahmad Z, ***Wong L-F**, Murphy D and Kasparov S (2001)* Nitric oxide mediates the baroreceptor reflex depression induced by angiotensin II in the nucleus of the solitary tract of rat. J Physiol 531(2): 445-58

***Wong L-F**, Wells S and Murphy D (2000)* Gene transfer strategies for the physiologist. Exp Physiology 85(6): 735-45

Paton JFR, ***Wong L-F**, Murphy D and Kasparov (2000)* Angiotensin II induced baroreceptor reflex depression in the nucleus tractus solitarius (NTS) is mediated by nitric oxide (NO). FASEB J 14: 460.8

***Wong L-F**, Edwards E, Uney J, Harding T, Murphy D, Kasparov S and Paton JFR (2000) In vivo* gene transfer demonstrates a role for camp-dependent protein kinase (PKA) in the nucleus of the solitary tract (NTS) for modulating the peripheral chemoreceptor reflex. J Physiol (Lond) 523: 259P-60P

Referees

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Nicholas Mazarakis PhD - Director – Neurobiology, Oxford BioMedica

Dr Nicholas Mazarakis obtained his B.Sc. in Applied Biology from the University of East London and his Ph.D. in Biochemistry at King's College London, where he worked on mammalian molecular genetics. He subsequently worked through two postdoctoral fellowships at the National Institute for Medical Research, Mill Hill, in the departments of Gene Structure and Expression, and Neurobiology. His research spanned neuronal gene expression, immortalisation studies in transgenic mice, gene transfer in neural cells and transplantation in animal models of spinal injury. He then joined the department of Paediatrics and Neonatal Medicine at the Royal Postgraduate Medical School in London, where he investigated the role of apoptosis in neonatal brain injury. In 1997, he was recruited by Oxford BioMedica, a start-up company specialising in gene therapy, where he is currently Director of Neurobiology.

Together with his colleagues at Oxford BioMedica, he has developed a novel lentiviral vector system based on the non-primate lentivirus -Equine Infectious Anaemia Virus (EIAV) and has successfully demonstrated that it mediates efficient gene transfer to neuronal cells both *in vitro* and *in vivo*. Using these vectors he has developed a dopamine replacement therapy for late stage Parkinson's disease (ProSavin) which is now in clinical development. In addition a collaboration with researchers at the Bristol Medical School has demonstrated and achieved long-term correction of an animal model of Diabetes Insipidus. He has also demonstrated that pseudotyping the EIAV vectors with the rabies virus envelope, allows retrograde axonal transport of the viral vector and expression in neurons distal to the site of injection, such as motoneurons after delivery into muscle. This has opened up the exciting opportunity of applying such an approach to the treatment of Motor Neuron Diseases and Spinal Injuries.

Key Publications:

Mitrophanous K, Yoon S, Rohll J, Patil D, Wilkes F, Kim V, Kingsman S, Kingsman A, **Mazarakis N.** (1999) Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Ther 6: 1808-18*

Mazarakis N.D., Azzouz M, Rohll J.B., Ellard F.M., Wilkes F.J., Olsen A.L., Carter E.E., Barber R.D., Baban D.F., Kingsman S.M., Kingsman A.J., O'Malley K., and Mitrophanous K.A. (2001) Rabies virus glycoprotein pseudotyping of retroviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. *Hum Mol Genet 10: 2109-2121*

Mimoun Azzouz, Enca Martin-Rendon, Robert D. Barber, Kyriacos A. Mitrophanous. Emma E. Carter, Jonathan B. Rohll, Susan M. Kingsman, Alan J. Kingsman, and **Nicholas D. Mazarakis**. (2002) Multicistronic Lentiviral Vector-Mediated Striatal Gene Transfer Induces Sustained Transgene Expression, Dopamine Production and Functional Improvement in a Rat Model of Parkinson's Disease. *J Neurosci 22: 10302-10312*

Alison S. Bienemann, Enca Martin-Rendon, Anna S. Cosgrave, Colin P.J. Glover, Susan M. Kingsman, Kyriacos A. Mitrophanous, **Nicholas D. Mazarakis** & James B. Uney. (2002) Long-Term Replacement of a Mutated Non-Functional CNS gene: Reversal of Hypothalamic Diabetes Insipidus Using a Lentiviral Vector Expressing Arginine Vasopressin. *Submitted*